Isolation of a New Glycoprotein-a and a γG-Globulin from Individual Cow Milks*

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ABSTRACT: A new protein, tentatively named glycoprotein-a, has been isolated from bovine milk. It shows a single band by gel electrophoresis at pH 4.3; however, on electrophoresis at alkaline pH, several closely spaced bands, attributable perhaps to genetic polymorphs, are evident. Glycoprotein-a has a sedimentation coefficient of 4.0 S and a minimum molecular weight of the order of about 48,000 based on the presence of a single methionine residue per molecule. A milk γG-globulin has also been isolated and characterized. Although it is closely related to milk pseudoglobulin in most of the chemical and serological properties investigated, differences in behavior in gel electrophoresis were observed. Comparisons of properties of γG-globulins prepared from bovine colostrum and blood indicate virtual identity of these proteins; minor chemical and immunological differences between these and the milk γG-globulin and pseudoglobulin were found.

Immune proteins from cow's milk and colostrum were isolated by classical methods of fractionation with ammonium sulfate by Smith (1946, 1948). Smith prepared and characterized euglobulin and pseudoglobulin from colostrum and milk and compared the properties of the purified proteins with those of immune globulins isolated from bovine blood. He found that all of these globulins were closely related, immunologically, but that they were not identical in physical and chemical properties.

We have applied the more refined methods now available for fractionating proteins to a reinvestigation of the proteins of the γ-globulin[1] fraction of cow's milk. In so doing, a new glycoprotein was found in

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[1] We follow the nomenclature for human immunoglobulins proposed in *Bull. World Health Organ. 30,* 447 (1964). γG-Immunoglobulin (or γG-globulin) denotes that class of immunoglobulins often designated as 7S γG-globulin.

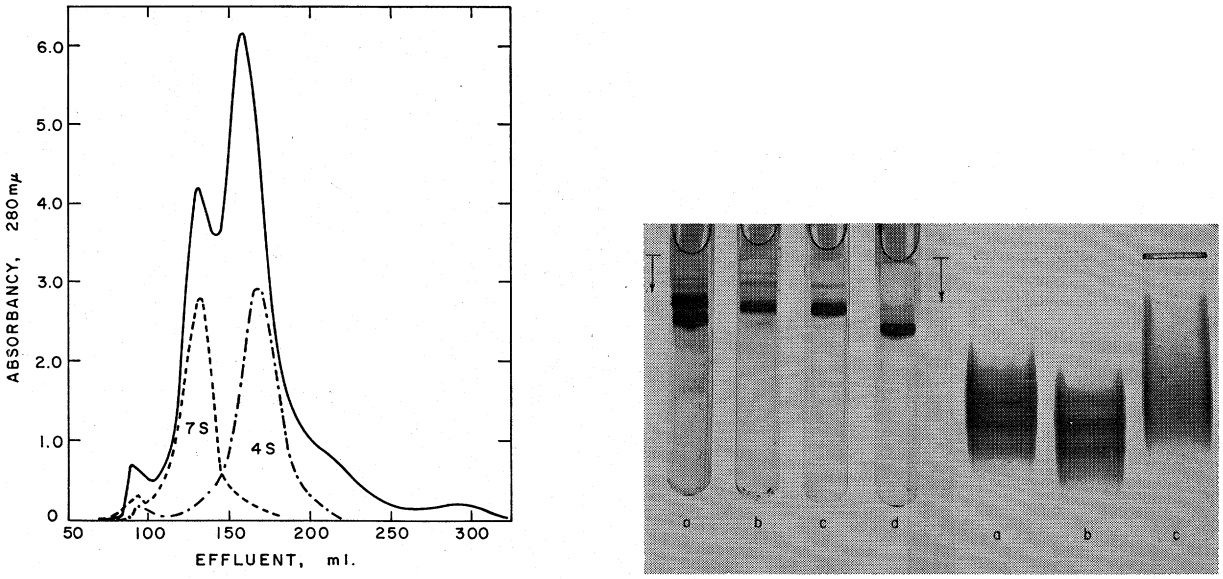

FIGURE 1: Fractionation, disc electrophoresis, and vertical gel electrophoresis. (A) (left) Fractionation of milk γG-globulin and glycoprotein-a on Sephadex G-200 column. The dashed line shows γG-globulin (7 S) after the third pass on the resin and the dot-dash line represents the glycoprotein-a. (B) (middle) Disc electrophoresis at pH 4.3, 1 hr at 60 ma: milk fraction IF-2 (a), protein in first minor peak (b), γG-globulin (c), and glycoprotein-a (d); the direction of arrow is toward the cathode. (C) (right) Vertical gel electrophoresis at pH 9.1, 5% gel: milk fraction IF-2 (a), glycoprotein-a (b), and γG-globulin (c); the direction of the arrow is toward the anode.

this fraction of milk, and purified γG-globulins were prepared from both milk and colostrum. For purposes of comparison, milk pseudoglobulin, as characterized by Smith, and bovine blood γG-globulin were also prepared. Although pseudoglobulin was obtained from mixed herd milk, the other milk proteins were isolated from milks of individual cows so that possible genetic polymorphisms might be detected. This report describes the methods used for the isolation of the new glycoprotein and of the γG-globulins, some of the physical and chemical characteristics of the proteins, and some comparisons of the γ-globulins from milk, colostrum, and blood.

Experimental Section

Isolation of the Proteins. γG-GLOBULIN AND GLYCOPROTEIN-a. Milk from individual cows was fractionated and chromatographed as previously described (Groves, 1965). Whey-F protein, in 0.005 M phosphate (pH 8.2), was first chromatographed on DEAE-cellulose, and fraction 1F, not retained on the column, was next chromatographed on phosphocellulose. The 1F-2 fraction, which was eluted from phosphocellulose with 0.1 M sodium phosphate at pH 6.0, moved as a single peak in the Perkin-Elmer[2] electrophoresis apparatus; its mobility was -1.26×10^{-5} cm²/v sec in 0.10 ionic strength Veronal buffer (pH 8.5). However, two components were demonstrable in the fraction by disc electrophoresis at acid pH and by ultracentrifugation at pH 7. The two components with sedimentation coefficients of 4 and 7 S were separated by gel filtration as follows. A 2 × 70 cm column was prepared with Sephadex G-200 previously washed and equilibrated overnight at 3° with 0.1 ionic strength sodium phosphate (pH 7.0). The protein (100–300 mg), dissolved in a minimum amount of buffer (1–2 ml), was carefully layered on the column and was eluted with the same buffer at a rate of about 8 ml/hr, as illustrated in Figure 1A. The fractions in the symmetrical part of the peaks were combined for refractionation. Usually three such gel filtrations were needed for adequate separation of the proteins. Yields of the two proteins prepared by this method were approximately 50 mg of glycoprotein-a and 30 mg of γG-globulin/l. of milk.

PSEUDOGLOBULIN. Pseudoglobulin was prepared from mixed herd milk by the method of Smith (1946) and deionized by mixed-bed resin according to Dintzis (1952).

COLOSTRUM γG-GLOBULIN. The γG-globulin was isolated from skim colostrum of an individual cow. The colostrum was first diluted with an equal volume of water to facilitate the precipitation of casein; otherwise, the fractionation procedure already described was followed. It is interesting to note that no glycoprotein-a was found in the 1F-2 fraction of colostrum.

BLOOD γG-GLOBULIN. γG-Globulin (bovine, Cohn's fraction II) was purchased from Nutritional Biochemi-

[2] It is not implied that the U. S. Department of Agriculture recommends the above company or its product to the exclusion of others in the same business.

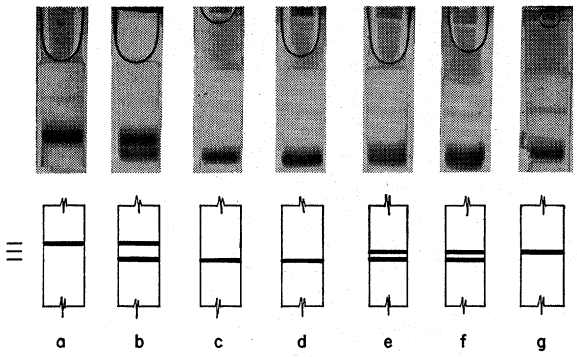

FIGURE 2: Disc gel electrophoresis at pH 4.3 for 1.5 hr at 60 ma. Pseudoglobulin (a), blood γG-globulin + pseudoglobulin (b), blood γG-globulin (c), blood γG-globulin + colostrum γG-globulin (d), milk γG-globulin (e), blood γG-globulin + milk γG-globulin (f), and milk γG-globulin (g).

cals Corp.[2] and fractionated like the milk protein on DEAE-cellulose, phosphocellulose, and Sephadex.

Reduction and Alkylation of Pseudoglobulin. Pseudoglobulin (58 mg) was reduced and alkylated following the method of Fleischman *et al.* (1962) for human and horse γG-globulin. After reduction and alkylation the pH was adjusted to 8 by the addition of solid Tris. The solution was dialyzed against 1 l. of 0.1 M sodium chloride to which 25 ml of Tris-HCl buffer was added, and then against 1 M acetic acid at 3° before lyophilization.

In another experiment 6.0 M urea was included in the reduction and alkylation. On dialysis of this product against acetic acid, the solution became very turbid at first but then cleared.

Gel filtration of the reduced and alkylated protein on a 2 × 70 cm column of Sephadex G-100 equilibrated with 1 M acetic acid gave an elution pattern like that shown by Fleischman *et al.* (1962) with a major A fraction (heavy chain) and a minor B fraction (light chain), the latter amounting to about 20% of the total. Pseudoglobulin reduced and alkylated in urea showed, on gel filtration, only a broad, single peak with much trailing, indicating strong interaction between the fully reduced chains.

Gel Electrophoresis. Disc electrophoresis was carried out at pH 4.3 by the method of Reisfeld *et al.* (1962) in the Canalco[2] apparatus with 12 tubes/run. Some runs were also made using 8 M urea solutions. For vertical acrylamide gel electrophoresis at pH 9.1, the Raymond system (Raymond and Nakamichi, 1962) was used, with a gel concentration of 5%.

Ultracentrifugation. Sedimentation experiments were made in the Spinco[2] Model E ultracentrifuge at 59,780 rpm in 0.1 ionic strength phosphate (pH 7.0) at 1% protein concentration, unless otherwise noted.

Carbohydrate Determinations. Hexose was determined by the reaction with orcinol (Winzler, 1955), with a 1:1 mixture of lactose and mannose as a standard. Weighed samples of the dialyzed and lyophilized proteins, corrected for moisture content, were used for these analyses.

Amino Acid Analysis. Proteins were hydrolyzed and analyzed automatically for amino acids by techniques described by Moore and Stein (1963). Duplicate determinations were made on samples hydrolyzed 24, 72, and 96 hr. Significant changes in the amounts of valine and isoleucine released with increasing times of hydrolysis were not observed. Glycoprotein-a was also oxidized by performic acid, hydrolyzed, and analyzed according to Moore (1963).

Results

Gel Electrophoresis of the Proteins. Figure 1B shows patterns from disc electrophoresis at pH 4.3 of milk γG-globulin (c) and glycoprotein-a (d) following fractionation on Sephadex, and of the original crude fraction 1F-2 (a). The pattern for purified γG-globulin demonstrates it to be free of glycoprotein-a; it contains, however, a small amount of a "slower moving protein" which is also evident in a and b. Pattern b represents the protein which emerges in the first small peak seen in Figure 1A; this fraction consists of γG-globulin as the main component, the slower moving protein, and small amounts of other proteins. Only traces of γG-globulin are detectable in the purified glycoprotein-a (Figure 1B, pattern d).

There is reason to believe from the ultracentrifugation experiments that the slower moving protein evident in Figure 1B, patterns a–c, represents higher molecular weight γ-globulin. A faint band with similar mobility is also present in the electrophoretic pattern of our preparation of pseudoglobulin (Figure 2a).

Rather different patterns are obtained when fraction 1F-2, γG-globulin, and glycoprotein-a are subjected to vertical gel electrophoresis at pH 9.1 (Figure 1C). Under these conditions, γG-globulin appears as a broad, diffuse area without well-defined bands (Figure 1C, pattern c). Glycoprotein-a gives four to five equally spaced bands of which the three most prominent may be distinguished in Figure 1C, pattern b. On examination of glycoprotein-a samples prepared from milks of individual cows, differences in mobilities of these three bands were observed. Some preparations showed a stepwise displacement of the entire series of bands to the extent of one or two bands. Whether these observations might be attributable to genetically controlled polymorphism is presently under investigation. It will be recalled that only a single, major band is visible in these preparations when they are run in acid gels by disc electrophoresis (Figure 1B-d).

Results of comparisons of the behavior of different γG-globulin samples in disc electrophoresis at pH 4.3 are shown in Figure 2. The relative position of the main bands may be seen more easily in the schematic representation. Pseudoglobulin (a) is slower in mobility than blood γG-globulin (c), as illustrated by (b) representing a mixture of the two. However, a mixture of the γG-globulins from blood and colostrum gave a

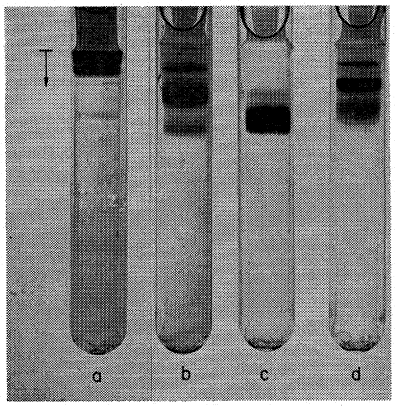

FIGURE 3: Disc electrophoresis at pH 4.3, 8 M urea, for 1 hr at 60 ma of pseudoglobulin reduced, alkylated, and fractionated on Sephadex. Unreduced pseudoglobulin (a), A fraction (b), B fraction (c), and psuedoglobulin reduced and alkylated in the presence of urea (d).

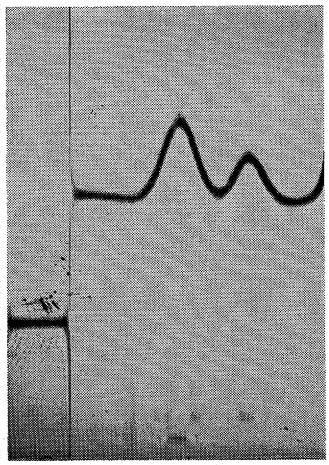

FIGURE 4: Ultracentrifugation pattern after 64 min of milk fraction IF-2 containing milk γG-globulin and glycoprotein-a before resolution on Sephadex; direction of sedimentation, left to right.

single band (d). The γG-globulins isolated from milks of individual cows show at least three types: a slow band (g) that moves slightly faster than pseudoglobulin (a); a fast band (not illustrated) with mobility similar to that of blood γG-globulin; and two bands (e) corresponding in mobility to the slow and fast bands. Pattern f, which resembles e, represents a mixture of blood γG-globulin and a slow milk γG-globulin. With γG-globulins that give two bands the ratio of slow to fast varies somewhat, depending on the cow.

Reduction and Alkylation Experiments. In Figure 3 are shown disc electrophoretic patterns in 8 M urea of the A and B fractions obtained from gel filtration of reduced and alkylated pseudoglobulin. Pattern a is that of the original, unreduced pseudoglobulin. Fraction A (pattern b) consists of the heavy chain (the principal middle band), some light chain (the fastest band), and an unidentified component (the slowest band). Fraction B (pattern c) is almost pure light-chain material. When the same procedure of reduction and alkylation was applied to pseudoglobulin in the presence of urea, gel filtration did not result in any separation of components, as already mentioned. Pattern d is representative of all the product in the single, broad peak. Evidently, no new chains resulted from reduction in the presence of urea, the number of bands being the same in d as in b and c together. However, the mobilities of the bands in d are somewhat slower. These results are in accord with the findings of Cohen (1963) on human γG-globulin.

Preliminary experiments on the reduction and alkylation in urea of small protein samples (0.5 mg) followed by disc electrophoresis without prior fractionation on Sephadex indicate a close similarity between pseudoglobulin and slow γG-globulin in that the light chain is well separated from the heavy chain. However, the γG-globulins from colostrum and blood behave somewhat differently from the others; they give rise to a diffuse, faster moving area for the light chain, not clearly separated from the heavy-chain band. The relative mobility of the heavy chain appears to be similar for all the globulins examined in this investigation. In the case of human serum and colostrum γ-globulins, recent comparisons of the electrophoretic behavior of the isolated heavy and light chains have shown striking differences in the relative mobility of the heavy chain (Rejnek *et al.*, 1966).

Glycoprotein-a was also run through the foregoing procedures with the thought that cleavage of the molecule at interchain disulfide bonds might result in the formation of new bands in disc electrophoresis. None were found so that it may be inferred that interchain disulfide linkages are not present in this protein. It was observed, furthermore, in these experiments that the mobility of reduced and alkylated glycoprotein-a was similar to, but not identical with, that of the heavy chain of the γG-globulins.

Ultracentrifugation. Figure 4 shows the sedimentation pattern at 24° of fraction 1F-2, prepared from the milk of one cow, before filtration on Sephadex; sedimentation coefficients, $s_{20,w}$, of 4.36 and 7.28 S were determined for the lighter component, glycoprotein-a, and the heavier γG-globulin, respectively. At 1°, the corresponding coefficients are 3.97 and 6.72, about 7% lower for both proteins. Fraction 1F-2 from another cow gave similar values of 4.06 and 6.80 S at 1°.

Purified glycoprotein-a, when centrifuged in 1% concentration at 2°, sediments with a coefficient of 4.02. Identical values were obtained from 0.5, 1.5, and 2% solutions, indicating that the coefficient for this protein is not dependent on concentration.

The sedimentation coefficient for purified γG-globulin was found to be 6.81 at 1°. Our preparation of pesudoglobulin gave a value of 6.54 S at 23° for the major component, and for a minor, heavier component (estimated as about 5% of the total protein), a value

of 9.81 S; the latter is presumed to be the slower moving protein mentioned in the preceding section. These values are in approximate agreement with those obtained by Smith and Brown (1950) for milk pseudoglobulin and euglobulin centrifuged in 0.15 M NaCl at pH 6.2 at about 25°; they, too, found a minor component with a sedimentation coefficient of about 10 S to be present in their preparations.

Carbohydrate and Amino Acid Analysis. Application of the orcinol reaction to glycoprotein-a and to milk γG-globulin gave values of 3.12 and 1.14% hexose, respectively. The latter figure is comparable to 0.93% found by Nolan and Smith (1962) in a preparation of γG-globulin isolated from the colostrum of a hyperimmunized cow. In addition to hexose, it is likely that hexosamines are present in glycoprotein-a, for amino acid chromatograms of 24-hr hydrolysates of this protein show two peaks following phenylalanine in positions corresponding to the effluent volumes of glucosamine and galactosamine (Walborg *et al.*, 1963); with longer times of hydrolysis, these peaks disappeared.

The amino acid composition of the γG-globulins and of glycoprotein-a, calculated as micromoles of amino acid and normalized to glycine, are shown in Table I. The comparisons which follow are equally valid if molar ratios are calculated with phenylalanine assigned the value of one. The relatively high content of hydroxyamino acids is typical of γG-globulins. Like human and rabbit γG-globulins (Crumpton and Wilkinson, 1963), the bovine γ-globulins also contain proportionately large amounts of valine, proline, leucine, glutamic acid, aspartic acid, and lysine, while methionine, histidine, isoleucine, and phenylalanine are present in relatively small amounts.

The content of basic amino acids in the blood and colostrum γG-globulins is significantly higher than that in pseudoglobulin and slow milk γG-globulin, but otherwise no great differences are apparent. Fast milk γG-globulin was not available for analysis.

Glycoprotein-a contains much less serine and threonine than γG-globulin. However, it is also relatively rich in valine and lysine and poor in methionine, histidine, isoleucine, and phenylalanine. Because the methionine content was so low, special hydrolysates, described in the table, were analyzed for this amino acid and the value shown is of the same order as a determination as methionine sulfone, following performic acid oxidation of the protein. On the assumption that the glycine to methionine ratio listed in the table is approximately correct, a minimum molecular weight for glycoprotein-a was calculated. This is based on a single methionine residue and the summation of the appropriate numbers of other amino acid residues derived from the molar ratios. The numbers of amino acid residues rounded to the nearest integer are shown in the table. The calculated molecular weight of 48,000 is admittedly an approximation but it is in the range that might be expected from the sedimentation coefficient of 4.0 S. Neither tryptophan, which was not determined, nor carbohydrate was considered in the calculation; their inclusion would increase the minimal molecular weight. Unfortunately, data necessary for calculation of molecular weight from the sedimentation experiments are not available.

Discussion

The isolation from the immune globulin fraction of whey of glycoprotein-a, a protein differing in composition and properties from γG-globulin and other, previously characterized γ-globulins, was unexpected, though perhaps not too surprising in view of the extreme complexity of this fluid. Despite the observed differences in carbohydrate content, amino acid composition, sedimentation coefficient, and electrophoretic behavior before and after reduction and alkylation, it is conceivable that glycoprotein-a constitutes a fragment of γG-globulin. The most convincing evidence that this is not the case is provided by the results of immunochemical studies, to be published elsewhere by E. J. Coulson. Dr. Coulson compared milk γG-globulin and glycoprotein-a by double diffusion in a four-well Ouchterlony plate in which the antigens and respective antisera occupied opposite wells. The major precipitin lines of the two systems crossed each other without interference. Thus, there was no antigenic relationship between the two preparations. The question regarding the occurrence of genetically controlled polymorphism of glycoprotein-a has been raised in this investigation but cannot yet be fully answered without considerably more experimental data.

The milk γG-globulin isolated in this work is closely related to milk pseudoglobulin as prepared by Smith's method. Amino acid compositions and sedimentation coefficients are very similar. The proteins appear to be serologically identical when they are diffused together against either of their respective antisera (E. J. Coulson, to be published). However, as the disc electrophoretic experiments have indicated, slow and fast forms of γG-globulin may be distinguished, suggesting the possibility of genetic polymorphism. It may also be noted that there is a small difference in mobility between the slow form and the even slower pseudoglobulin; this might be owing to the fact that the latter was not rigorously purified by column chromatography.

Our results also support the generally accepted, close relationship between, or identity of, the γG-globulins from bovine colostrum and blood. No significant differences in amino acid composition, in electrophoretic behavior, before or after reduction and alkylation, or in serological behavior were demonstrable.

When milk γG-globulin and pseudoglobulin as one pair of related proteins are compared with colostrum and blood γG-globulins as another, some minor differences are apparent. The difference in appearance of the disc electrophoretic patterns following reduction and alkylation, notably the relatively poor separation of the light chain in the case of the latter pair, has already been mentioned. Furthermore, the slow milk γG-globulin and pseudoglobulin migrate more slowly in disc electrophoresis at pH 4.3, and this difference in mobility may be a consequence of the lower content

TABLE I: The Amino Acid Composition of the Globulins and Glycoprotein-a.

Amino Acid	μmoles of Amino Acid/μmole of Glycine										Residues of Amino Acids/Mole of Methionine Glycoprotein-a
	Blood γG-Globulin	Std Dev	γG-Globulin (colostrum)	Std Dev	Pseudo-globulin	Std Dev	γG-Globulin, Slow (milk)	Std Dev	Glycoprotein-a	Std Dev	
Lys	0.753	0.042	0.811[a]	0.036	0.683[b]	0.017	0.696	0.011	0.682	0.035	27
His	0.228[a]	0.015	0.231[a]	0.009	0.201[b]	0.002	0.204	0.006	0.166	0.016	7
Arg	0.532[a]	0.019	0.539[a]	0.014	0.407[b]	0.007	0.469	0.011	0.540	0.027	21
Asp	1.102	0.018	1.173	0.014	1.052	0.011	1.064	0.009	1.205	0.018	47
Thr[c]	1.320	0.039	1.318	0.022	1.361	0.016	1.289	0.008	0.742[e]	0.007	29
Ser[c]	1.840	0.039	1.859	0.052	1.795	0.022	1.870	0.018	1.011	0.027	40
Glu	1.153	0.019	1.133	0.040	1.165	0.057	1.143	0.005	1.206	0.013	47
Pro	0.845	0.028	0.857	0.044	0.936[a]	0.053	0.844	0.015	0.576	0.024	23
Gly	1		1		1		1		1	1	39
Ala	0.755	0.006	0.722	0.007	0.726	0.005	0.722	0.009	0.639	0.007	25
$^1/_2$-Cys									0.465[d]	0.007	18
Val	1.356	0.012	1.341	0.036	1.248	0.026	1.267[a]	0.015	1.174	0.022	46
Met	0.124[e]	0.007	0.114[e]	0.011	0.093[e]	0.013	0.100	0.004	0 0256[f]	0.001	1
Ile	0.361	0.008	0.384	0.010	0.342	0.009	0.337	0.009	0.378[a]	0.002	15
Leu	0.894	0.021	0.884	0.023	0.911	0.033	0.850	0.009	0.733	0.007	29
Tyr	0.466[e]	0.003	0.468[e]	0.006	0.471[e]	0.001	0.471[a]	0.010	0.434[b]	0.006	17
Phe	0.367[a]	0.016	0.372	0.019	0.359	0.013	0.344	0.009	0.364[a]	0.014	14

[a] Average of five determinations. [b] Average of four determinations. [c] Values for threonine and serine are calculated for linear regression to zero time. For threonine in glycoprotein-a, only four points were used since the 72- and 96-hr values were in agreement. [d] Determined in duplicate as cysteic acid following oxidation by performic acid (Moore, 1963). [e] Average of two determinations. [f] Since content of methionine is low, about 8 mg of protein in duplicate was hydrolyzed 24 hr. For application to the column, hydrolysates were made to 5 ml and 4.00 ml was applied to the column for the methionine and 0.50 ml for the glycine determinations.

of basic amino acids characteristic of this pair; finally, subtle immunochemical differences between the two pairs have been observed by Coulson and will be described in detail in a separation publication.

In connection with these comparisons of the γG-globulins, and also in considering the possibility of the occurrence of polymorphs, genetically controlled, of the milk γG-globulins, the investigation of Murphy *et al.* (1964) is of particular interest. Murphy *et al.* prepared bovine γG-globulins from blood serum and from colostrum and separated them into so-called slow and fast globulins by means of column chromatography with DEAE-Sephadex. The globulins eluted at the front, at pH 8.0, were designated slow because of their relatively slow migration in electrophoresis at alkaline pH; the globulins eluted at higher salt concentration and at lower pH values were called fast because of higher mobility under the same conditions. Although the immune globulins from bovine serum and dry secretion whey (obtained during the nonlactation period) showed both the slow and fast components in similar amounts, colostrum contained very little slow material relative to the fast. Pierce and Feinstein (1965) also reported this unique selectivity of the mammary gland for the secretion of certain immune globulins.

In the present work, only that fraction of the immune proteins of milk not retained by DEAE-cellulose (0.005 M phosphate buffer, pH 8.2) was investigated. This fraction provided the starting material for subsequent purification on phosphocellulose and Sephadex, and, presumably, would correspond to the slow γG-globulin of Murphy which is present in relatively small amounts in colostrum and in larger amounts in blood serum. Similarly, in our preliminary fractionations of the colostrum whey proteins and of bovine blood γ-globulin on DEAE-cellulose, about 6 and 50%, respectively, of the total protein chromatographed were not retained by the absorbent. It should be emphasized that the milk γG-globulins designated slow and fast in the present report (for example, in Figure 2) *both* occur in this fraction. But it is clear from Smith's earlier investigations, from Murphy's work, and from our own experiments that the γG-globulin described here is only one of a number of the so-called immune proteins of milk and colostrum. Other γ-globulins, bound by DEAE-cellulose under the above conditions, can be eluted at higher ionic strengths and may be found, for example, in the 4F fraction of Groves (1965).

In summary, we conclude that glycoprotein-a is chemically and serologically distinct from the γ-globulin proteins with which it is associated in cow's milk. Because samples of glycoprotein-a of different electrophoretic mobilities have been isolated from individual milks, it is possible that the observed polymorphism of the protein is genetically controlled.

γG-Globulins from bovine colostrum and blood are very similar in their electrophoretic behavior before and after reduction and alkylation, amino acid composition, and serological properties. Milk pseudoglobulin and slow γG-globulin are also very similar in these respects. However, small differences between the two pairs are evident. Detection of slow and fast forms of milk γG-globulins indicates the likelihood of genetic control either of selectivity of transfer in the mammary gland or of synthesis of this protein.

Acknowledgments

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